PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/50, 33/96, 33/50
G01N 33/92 // G01N 33/574

A1

(11) International Publication Number: WO 92/08976

(43) International Publication Date: 29 May 1992 (29.05.92)

(21) International Application Number: PCT/NL91/00233

(22) International Filing Date: 19 November 1991 (19.11.91)

(30) Priority data:
615,597 19 November 1990 (19.11.90) US

(71) Applicant *(for all designated States except US)*: ONCO MEDICS B.V. [NL/NL]; Klaaskampen 1, NL-1251 KM Laren (NL).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : KATOPODIS, Nonda [US/US]; 10 Greens Cir., Stamford, CT 06903 (US).

(74) Agents: DE BRUIJN, Leendert, C. et al.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU+,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR DETERMINING LIPID BOUND SIALIC ACID IN PLASMA

(57) Abstract

The amount of lipid bound sialic acid in a blood plasma or serum sample may be determined by an improved method which may be automated involving the following steps to be performed simultaneously on the sample and a known standard: diluting with buffer; mixing the diluted sample; adding a mixture of a chlorinated lower alkyl alcohol; mixing, diluting with buffer and then treating by mixing further and centrifuging to yield a substantially clear upper phase; recovering the upper phase and adding to it a protein precipitating agent, mixing the resulting admixture; recovering the resulting precipitate, suspending the precipitate in a hydrolysis agent and determining the amount of lipid bound sialic acid present by comparing the optical density of the sample to the optical density of the standard. An improved standard for this method is provided as well.

+ See back of page

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

Method for determining lipid bound sialic acid in plasma.

BACKGROUND OF THE INVENTION

This invention concerns an improved method for the determination of lipid bound sialic acid in plasma or serum which is less expensive, less time consuming, less variable from sample to sample, and less dependent upon the skill and experience of the person performing the test. Additionally this invention concerns an improved test kit which contains a unique standard which will allow greater reproducibility of test results and more consistency throughout the world.

Much work has been done which indicates that elevated sialic acid content in blood sera of a patient is an indication of the presence of cancer. For example, U.S. Patent 4,146,603 to Davidson, et al. discloses a fairly complex series of procedures whereby elevated sialic acid content is a determinant with respect to cancer specific determinations.

MacBeth and Bekesi, Cancer Res. 22: 1170-1176 (1962), measured plasma glycoproteins and found galactose and mannose values were seen in breast cancer without metastases. Kloppel, et al., Proc. Natl. Acad. Sc. 74: 3011-3013 (1977), reported 2.5-fold increases of serum sialic acid glycolipids in mice bearing transplantable mammary carcinomas and 2-fold increases in human carcinoma patients. Kloppel, et al., Am. J. Vet. Res. 39: 1377-1380 (1978), also reported increases of sialic acid in 93% of 24 dogs; In Leukemia AKR/J mice, Lengle, J. Natl. Cancer Inst. 62: 1565-1567 (1979) found increased lipid bound sialic acid in their plasma and thymic lymphocytes. Lipid bound sialic acid levels were found increased in plasma and erythrocytes of humans bearing melanomas, Portouklian, et al., Biochem. Biophys. Res. Commun. 85: 916-920 (1978). Chromatographic separation and purification on columns was followed by evaluation on chromatoplates. Silver, et al., Cancer 41:1497-1499 (1978); Cancer Res. 39:5036-5042 (1979) have reported elevated serum sialic acid values in melanoma patients that were significantly related to the tumor burden. However, 36% of patients with observable tumors showed no elevated serum sialic acid. Hogan-Ryan, et al., Br. J. Cancer 41:587-592 (1980), reporting on total bound serum sialic acid in patients with breast cancer found elevations that corresponded with tumor stage.

One specific method over which the present invention is an improvement is disclosed in the American Association for Cancer Research Annual Meeting Proceedings, Vol 21, March 1980 as Abstract No. 728 by Katopodis, et al. Briefly, this method requires that a 100 µl plasma sample (reduced to 50 µl) be extracted with 6 ml of a chloroform/methanol mixture, (2 to 1, volume to volume ratio). The lipid extract is then partitioned with 0.2 of its volume of water. The aqueous phase is evaporated to dryness and the residue redissolved in water. The lipid bound sialic acid is then purified by trichloroacetic acid-phosphotungstic acid precipitation and, after the removal of the supernatant from the resultant precipitate, the precipitate is determined by the Svennerholm and Miettien method (Svennerholm, Quantitative Estimation of Sialic Acid, Biochem. Biophys, Acta. 24, pp. 604-611 (1957)).

Another specific method over which the present invention is an improvement is disclosed in Katopodis and Stock, U.S. Patent 4,342,567, issued August 3, 1982. This method is similar to the foregoing but requires only about 50 µl of sample rather than the 100 µl required by the prior art method. The drying step is eliminated and there is no use of trichloracetic acid. Phosphotungstic acid is used alone.

These specific methods suffer from a number of disadvantages including the following: the need for a precisely defined 44.7 µl starting sample; lipid bound sialic acid is lost during the tube inversion step creating reduced final values; precipitation of the lipid bound sialic acid with phosphotungstic acid is not complete, which is a particular problem when working with samples in which the amount exceeds normal values by only small amounts (e.g. early in cancer development); the rapidity of the test is limited by the 5 minutes waiting time after phosphotungstic acid addition and the cost of the test is not as low as is desirable.

Another method over which the present invention is an improvement is disclosed in U.S. Patent 4,748,128, issued May 31, 1988 (Katopodis). This prior method consists of the following steps:

(a) diluting a predetermined volume of a blood plasma or serum with distilled water to a volume about four times that of the predetermined volume;

(b) mixing the diluted sample for a suitable period of time to obtain a substantially homogeneous sample;

(c) cooling the mixed, diluted sample to about 0° to 5°C;

(d) adding to the cooled sample a mixture of a chlorinated lower alkyl hydrocarbon and a lower alkyl alcohol, the volume of the mixture added being about sixty times the predetermined volume of the blood plasma or serum sample, and the volume ratio of chlorinated hydrocarbon to alcohol in the mixture being about 2:1 and its temperature about 0° to 5°C;

(e) mixing the resulting admixture for a suitable period of time to dissolve matter present in the sample in the chlorinated hydrocarbon/alcohol mixture;

(f) diluting the admixture with deionized distilled water at a temperature from about 0° to 5°C, the volume added being about ten times the predetermined volume of the blood plasma or serum sample;

(g) treating the diluted admixture for a suitable period of time to permit formation of a substantially clear upper phase;

(h) separately recovering from the clear upper phase so formed a predetermined volume of the upper phase;

(i) adding to the predetermined volume of the upper phase an amount of a mixture of a protein-precipitating agent and an adsorbing material, the amount of mixture being effective to cause precipitation of the lipid bound sialic acid;

(j) mixing the resulting admixture;

(k) separately recovering the resulting adsorbed precipitate;

(l) suspending the precipitate in a suitable volume distilled water; and (m) adding to the suspended precipitate a volume of resorcinol reagent, mixing, boiling for 15 minutes, cooling for 10 minutes in an ice bath, centrifuging, adding a mixture of butyl acetate and n-butanol (85:15 v/v) in a volume about twice the volume of the resorcinol reagent, mixing, centrifuging, separating the organic layer, reading at 580 nm the extracted blue colour present in the organic layer, and determining the amount of lipid bound sialic acid by comparing the optical density reading obtained at 580 nm to a standard curve developed from a known sample of N-acetylneuraminic acid (NANA) under the same conditions and applying the formula:

$$\text{LSA (mg/100 ml plasma)} = (x \cdot 10^5\ \mu l)/y \cdot z\ \mu l \cdot 1000)$$

where x = NANA read from the standard curve, y = the volume of the upper phase recovered divided by the total volume of the entire upper phase and z = the predetermined volume of the blood plasma or serum sample.

The present invention provides an improved method for determining the amount of lipid bound sialic acid present in a sample or serum. The present

invention is more economical, more time efficient, more easily automated, and requires less labour and chemical reagents than the methods of the prior art. The procedure of the present invention differs significantly from known methods in that the present invention requires a much smaller sample of blood plasma or serum, eliminates the need to cool the mixture to a point below room temperature, and eliminates the need to use an adsorbing material. Moreover the present invention uses a unique standard which provides greater uniformity and better reproducibility from laboratory to laboratory and which enables the user to more easily and more accurately determine the level of sialic acid in the sample.

The present invention requires a plasma or serum sample of only about one quarter of that required by the prior art. The ability to use a smaller sample has important advantages. It facilitates obtaining a sample from children and elderly patients as only a prick of the finger is required rather than the drawing of blood from a vein. It allows many more tests to be conducted from a volume of blood plasma or serum. For example the present invention permits twice or even four times as many tests from a sample of plasma or serum than the procedure of U.S. Patent 4,748,128 (Katopodis). The ability to conduct the test on smaller samples is also important in the screening and testing of new drugs on mice and rats. For example, use of the smaller samples of the invention allows a mouse to be bled each day without disturbing its metabolism. This permits more accurate testing since the effect of a drug on a single mouse can be tracked rather than requiring the test to be conducted on many mice and then averaged.

The prior art requires facilities to cool the mixture to 0°C or below and requires the use of an adsorbing material made of siliceous material usually silica or silica gel. The present invention does not require ice making equipment which is expensive and not available in many laboratories around the world.

Significantly the present invention provides an improved procedure for determining the concentration of lipid bound sialic acid in a sample of human blood plasma or serum by comparing the sample with a known standard comprised of a similar substance, i.e. human or animal blood plasma or serum rather than a chemical, i.e. N-acetylneuramic acid (NANA) as in the prior art. It also eliminates the need to construct a standard curve for the standard as in the prior art. The improved procedure also eliminates the

deviation between the optical density found for the sample and the standard curve due to variations in the techniques employed in carrying out the test procedure.

SUMMARY OF THE INVENTION

The present invention provides a method for extracting sialic acid from a sample of human blood plasma or serum and determining the amount of lipid bound sialic acid present in the sample which includes the following steps:

a) diluting a predetermined volume of a blood plasma or serum sample with an aqueous buffer having a volume of about 1.5 to 2.5 times that of the predetermined volume of the sample;

b) mixing the diluted sample for a suitable period of time to obtain a substantially homogeneous sample;

c) adding to the sample a mixture of a chlorinated lower alkyl hydrocarbon and a lower alkyl alcohol, the volume of the mixture added being about 20-60 times the predetermined volume of the blood plasma or serum sample, and the volume ratio of chlorinated hydrocarbon to alcohol in the mixture being from about 1:1 to 4:1.

d) mixing the resulting admixture for a suitable period of time to dissolve lipid-bound sialic acid in the sample in the chlorinated hydrocarbon/alcohol mixture;

e) diluting the admixture with a buffer solution at about room temperature, the volume of buffer solution being about 6 to 10 times the predetermined volume of the blood plasma or serum sample;

f) mixing the diluted admixture for a suitable period of time to obtain a substantially homogeneous admixture and centrifuging the mixture to form a substantially clear upper phase;

g) recovering from the clear upper phase so formed a predetermined volume of the upper phase;

h) adding to the predetermined volume of the upper phase a mixture of a protein-precipitating agent and water, the amount of said mixture being effective to cause precipitation of the lipid bound sialic acid;

i) mixing the resulting admixture;

j) recovering the resulting precipitate;

k) suspending the precipitate in a hydrolysis agent;

l) determining the amount of lipid bound sialic acid present in the suspended precipitate and thereby the amount present in the blood plasma or serum sample.

The determination of the amount of lipid bound sialic acid in the sample is made by comparing the optical density of the sample to that of a known standard derived from human tissue, human blood or animal blood or a proteinous material. Animal blood is preferred because it is readily available and normally free from contamination causing infectious disease to humans. For the same reason, animal serum albumine is a preferred proteinous material.

The standard may be prepared using the following steps:

(a) obtaining a sample of blood plasma or serum from whole blood or a body tissue extract;

(b) diluting a predetermined volume of the sample with water;

(c) mixing the diluted sample to obtain a homogeneous sample;

(d) adding to the sample a lower alkyl alcohol;

(e) mixing the resulting admixture to dissolve matter present in the sample in the alcohol mixture;

(f) treating the admixture to form a supernatant and a precipitate;

(g) diluting the precipitate;

(h) determining the amount of lipid bound sialic acid present in the diluted precipitate or in the supernatant.

A volume of the standard equivalent to the volume of the sample being tested is treated exactly the same as, and simultaneously with, the sample according to the procedure set forth above for the sample.

The concentrate of lipid bound sialic acid in the sample is found by multiplying the optical density of the sample times the known concentration of lipid bound sialic acid of the standard and dividing the resulting value by the optical density of the standard.

The invention further provides an improved method of preparing a standard for having a known concentration of lipid bound sialic acid which comprises the following steps:

(a) dissolving a proteinous material in distilled water in an amount of about 3-15 g per 100 ml;

(b) optionally adding up to 0,5 g of N-acetylneuraminic acid to the solution of the proteinous material in water;

(c) determining the amount of lipid bound sialic acid present in the resulting mixture.

The improved procedure of the invention provides improved reproducibility of the test and improved accuracy of the test results in any laboratory because the test sample and the known standard are treated with exactly the same techniques. Thus variations between the test results for the sample and a standard which are due to variations in the procedures or techniques used in testing the sample are eliminated.

This invention also provides a method and kit for diagnosing cancer in a human or animal subject which comprises determining the amount of lipid bound sialic acid in a sample of a subject's blood plasma or serum and comparing the amount obtained with values obtained for subjects known to have cancer. This is course very important in human medicine. The method of the invention also, for example, permits a veterinarily important and easy distinction between a stomach cancer and an ulcer in cows.

Alternatively the method and kit of this invention may be used to regularly determine the amount of lipid bound sialic acid present in a subject's blood plasma or serum and thus to monitor the progress of therapy of a subject by comparing each amount so determined with amounts previously determined for the subject.

Another aspect of the invention concerns a method of diagnosing cancer in a human or animal subject which comprises determining the amount of lipid bound sialic acid in a sample of the subject's plasma or serum and comparing the amounts so determined with values obtained for normal subjects, normal meaning that X-ray or other analysis has shown a healthy condition, or alternatively, comparing the amount so determined with values obtained over a period of time for the same subject.

The invention also provides a method for monitoring the progression of cancer in a subject which comprises determining at regular time intervals the amount of lipid bound sialic acid in a sample of the subject's blood plasma or serum according to the method of the present invention and comparing the amount so determined with amounts previously obtained for the subject.

The method of the invention is also found to be suitable for early detection of recurrence of cancer up to 6 to 9 months, before the appearance of symptoms.

Furthermore, this invention provides an improved cancer diagnostic kit comprising a container with a mixture of a lower alkyl alcohol and chlorinated lower alkyl hydrocarbon; a container of protein precipitating agent; optionally a container of prediluted resorcinol reagent; optionally a container with a stabilized buffer solution and an organic solvent which is essentially water-immiscible such as a mixture of butyl acetate and butanol; a container of a standard having a known concentration of lipid bound sialic acid; and instructions enabling the user of the kit to conduct the test and compare it to the standard provided to determine the amount of lipid bound sialic acid in the sample without variations between the sample and the standard resulting from the techniques or procedures used by the person conducting the test.

DETAILED DESCRIPTION OF THE INVENTION

The amount of lipid bound sialic acid in a sample of human blood plasma or serum may be determined and the amount so determined used as a diagnostic indicator of cancer. A preliminary step to the method is to obtain a sample to be tested. The sample will typically be recovered from whole blood drawn from a subject and treated using methods which are well known and described in the prior art. See, for example, Katopodis, U.S. Patent 4,748,128. The present method can be performed using a very small volume of sample, for example as little as 10 µl of plasma or serum.

The initial step of the method of the present invention is to dilute a predetermined volume of a blood plasma or serum sample with buffer solution. The buffer solution preferably has a pH value of about 5.5-5.6 and may contain usual buffering agents. The volume dilution is preferably about two times the volume of initial plasma sample. Thus, if the initial plasma or serum sample is 25 µl in a small tube or container, the amount of buffer added may be about 50 µl to produce about 75 µl of diluted sample, i.e. about three times the volume of the initial sample.

It should be understood that where the term "about" is used in the present specification in connection with a speficied numerical value, in general a range of values from 50% to 200% of the specified numerical value, and in particular a range from 80% to 125% of the numerical value is meant.

The diluted sample is mixed, e.g. by vortexing, for a suitable time to

obtain a substantially homogeneous sample, e.g. for at least 2 seconds.

The present invention eliminates the need for cooling to 0°C as required in the prior art thus enabling many laboratories in third world nations which do not have ice making equipment to use this invention.

A mixture of a chlorinated lower alkyl hydrocarbon and a lower alkyl alcohol in which the volume ratio of chlorinated hydrocarbon to alcohol is between about 1:1 and 4:1, preferably about 2:1, is then added to the sample. The volume of the chlorinated hydrocarbon and alcohol mixture added is preferably about forty times the original, i.e. predetermined, volume of the plasma sample and its temperature is room temperature. Thus, if the original sample volume is 25 µl, then the volume of mixture added is about 1.0 ml. Suitable chlorinated hydrocabrons include chloroform, dichloromethane, dichloroethane, dichloropropane, trichloroethane and tetrachloromethane, chloroform being presently preferred. The lower alkyl alcohol may be methanol, ethanol, propanol, butanol, sec-butanol, isopropyl alcohol, isobutyl alcohol or isoamyl alcohol. The greater the number of carbon atoms in the alcohol, the less effective the mixture is in terms of lipid bound sialic acid extraction as opposed to total sialic acid extraction. Therefore, the preferred alcohols are methanol and ethanol and most preferred is methanol, since the other alcohols extract higher amounts of total sialic acid and other contaminants, and therefore reduce the diagnostic value of the test.

The resulting admixture is then mixed for a suitable period of time to dissolve matter present in the sample in the chlorinated hydrocarbon/alcohol mixture, by gentle inversion. Mixing by vortexing, as in the prior art, produces an emulsion which is very difficult to separate. The admixture is then diluted with buffer, at room temperature, the volume added being about eight times the predetermined volume of the blood plasma or serum sample. Thus, if the original plasma sample was 25 µl and the amount of chlorinated hydrocarbon:alcohol mixture was 1.0 ml, the amount of buffer added would be about 0.2 ml.

The diluted admixture is then treated, first by mixing the diluted admixture for a suitable period of time, e.g. by gentle inversion for at least 30 seconds. Vortexing as in the prior art may adversely affect the test results. The mixture is then centrifuged e.g. for at least about five

minutes at about 3500 rpm to yield a substantially clear upper phase.

A predetermined volume of the upper phase is then separately recovered from the substantially clear upper phase so formed, preferably by removing the upper phase from the lower phase and discarding the latter. The predetermined volume so recovered will depend upon the volume of the original plasma sample. Thus, if the original, i.e. predetermined, plasma volume is about 25 µl, the volume of upper phase will be about 0.5 ml. The predetermined volume of the upper phase which is separately recovered will depend upon the convenience of removing a volume of the upper phase without disturbing the interface or other material in the tube. Preferably, the predetermined volume of the upper phase which is separately recovered is about 200 µl, allowing to recover another 200 µl volume later if necessary.

To the predetermined volume of the upper phase there is added an amount of a protein-precipitating agent, the amount added being effective to cause precipitation of the lipid bound sialic acid. Suitable protein-precipitating agents include phosphotungstic acid, trichloroacetic acid, ammonium sulfate or mixtures thereof (e.g. 90% phosphotungstic acid; 10% trichloroacetic acid), preferably an aqueous phosphotungstic acid solution (1:3 wt/vol). The invention eliminates the need for the addition of adsorbing materials such as silica and silica gel as required by the prior art.

The resulting admixture is then mixed, e.g. by vortexing briefly (at least 3 seconds), and the resulting precipitate is recovered, e.g. by centrifugation for at least 3 minutes at a speed of about 1300-1600 rpm (about 250-400 x g), and discarding the supernatant. The precipitate is then suspended in a suitable volume of resorcinol reagent, e.g. 0.5 ml, which causes hydrolysis of the sialic acid complex and formation of a chromofore. A preparation of the resorcinol reagent is decribed in US-A-4,748,128; the stock solution of resorcinol reagent is preferably diluted 70:30 to 50:50, preferably about 60:40, with water before use. The suspension is then treated by mixing, heating to 90°C or higher, preferably 95-110°C, for at least 10 minutes. The heating temperature and time depend on the strength of the resorcinol reagent. Heating can be performed by boiling for 15 minutes. The hydrolysis can also be done using a heating block suitable for 20 or more test tubes. In this case the block is adjusted to about 100-110°C and the samples are hydrolysed for 10 to 15 minutes. Heating may also be carried out in an oven or by other means.

Then the mixture is cooled for at least about 5 minutes, adding about twice said suitable volume, e.g. 1.0 ml, of a suitable organic solvent which is essentially immiscible with water selected from alkyl alcohols, alkanoic acid esters and their mixtures, for example pentanol or a mixture of an alkyl alcohol and an alkyl acetate or propionate. A mixture of butyl acetate and butanol (85:15 v/v) has been found to be a suitable solvent. After mixing, centrifuging for at least 5 minutes at above about 2500 rpm, separating the organic layer, reading at 580 nm the extracted blue colour present in the organic layer, the amount of lipid bound sialic acid is determined using the optical density of a known standard which is treated exactly the same as the sample, and simultaneously therewith, and applying the formula:

$$\text{LSA (mg/100 ml plasma)} = \frac{A \times B}{C}$$

where A = the known concentration of lipid bound sialic acid (LSA) in the standard; B = the optical density of the sample; and C = the optical density of the standard.

Instead of 1.0 ml of the mixture of butyl acetate and butanol, 0.5 ml of same mixture can be added to the tube, which will increase the optical density to about 70%. In this case a Micro-chem photometric analyser can be used (Source Scientific System Inc.) for example or other.

The increase of optical density will result in a more accurate reading of the final colour and reduce the necessary volume to 50%.

Another important aspect of the present invention is the method for preparing the standard used in the foregoing procedure. The standard may be human blood plasma or serum, animal blood plasma or serum, or an extract from animal or human tissue. Animal blood plasma or serum is preferred because animal blood is inexpensive, is readily available in large quantities and is normally free of bacterial or viral contaminations. The plasma or serum is obtained by centrifuging the whole animal blood at 2500 rpm for 10 minutes at room temperature. 100 ml of serum or plasma is extracted in the normal manner well known in the prior art. 100 ml of either the plasma or serum is used to prepare the standard. 100 ml of plasma or serum is transferred to a large container and mixed with an equal volume of distilled water. The mixture is mixed vigorously for about 5 minutes. To this mixture is added 500 ml of alcohol, preferably methanol, and the mixture is vigorously mixed for an additional 5 minutes. The mixture is then transferred to centrifuge tubes and centrifuged at 2500 rpm for 10 minutes

at room temperature. The supernatant is transferred to a flask for further treatment as described below. The precipitate is suspended in 100 ml of distilled water. This solution is the standard.

Each 1 ml or 0.5 ml (depending on the particular kit) of the standard is transferred to a separate vial and lyophilysed to a dry powder by procedures well known in the art. One ml or 0.5 ml respectively, of a buffer solution is used to reconstitute the standard for analysis. The concentration of lipid bound sialic acid in the standard is determined by use of a standard curve developed from a standard sample of N-acetylneuraminic acid (NANA). Where reference is made in the specification to N-acetylneuraminic acid, one of the other sialic acids may also be used, although less preferentially. It should be understood that the collected supernatant may also be used for the preparation of the standard. This is done by condensing the supernatant to 1/10 of its volume by rotary evaporation. This volume of the supernatant is then purified by extraction with a chloroform-methanol mixture and partitioned with water.

Alternatively, the standard may be prepared using: 1) animal serum, 2) proteinous material 3) NANA.
In order to facilitate the precipitation of the lipid bound sialic acid with phosphotungstic acid, an amount of a proteinous material is introduced for the preparation of the standard. Among a variety of proteinous materials, proteins having a molecular weight of about 10,000 to 100,000 are preferred. In particular, bovine serum albumine (BSA) may be used. Thus an aqueous solution of 3-15% (wt/vol.) of protein, such as bovine serum albumine (BSA) is prepared and introduced to the standard. Proteins having a molecular weight outside the molecular weight range mentioned above may also be used with appropriate adaptation of their concentration.

Since the amount of lipid bound sialic acid in normal bovine serum is very low, an extra amount of NANA (commercial standard) may be used. This commercial standard NANA is incorporated in the standard of the test which is then treated the same way as the unknown samples. This way by varying the amount of NANA in the BSA solution a series of concentrations of standards or controls can be established.

Example I:

1) Preparation of BSA reagent (bovine serum albumine):

3-15 g of BSA is dissolved by stirring in 100 ml of distilled water at room temperature. The solution is kept refrigerated.

2) Preparation of NANA-BSA reagent:

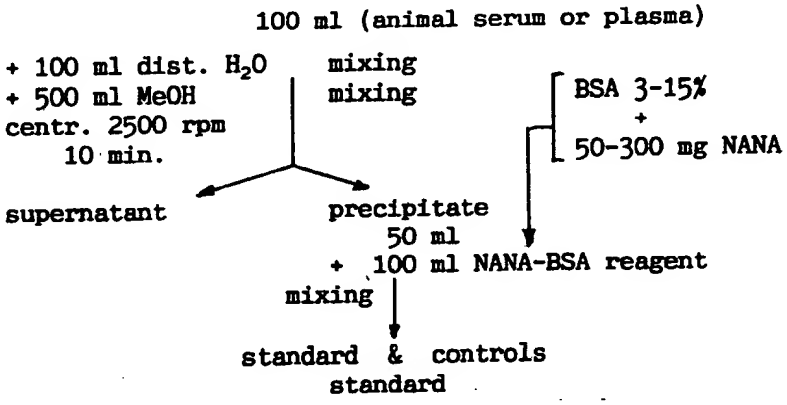
50-300 mg of commercial NANA standard (N-acetylneuraminic acid) is dissolved by stirring in 100 ml of BSA solution.

3) Preparation of the standard and controls of the kit:

100 ml of fresh animal (or human) serum or plasma is diluted with 100 ml of distilled water. After mixing 500 ml of methanol is added to the diluted serum and after mixing the mixture is centrifuged to 2500 rpm for 10 min. The precipitate (about 50 ml) is mixed with 100 ml of NANA-BSA reagent. This is the standard and controls of the kit. Depending on the number of tests in the kit, 0.3-0.5 ml is transferred to small vials and lyophilysed to a dry powder of standard and control.

The day of the analysis of the samples the vials are reconstituted with a proportional amount of buffer and they are used as standard and controls.

SCHEME



A further alternative method of preparing standard and controls uses only a proteinous material, such as commercial bovine serum albumin (BSA). In this method bovine or human serum is not used at all. NANA may or may not be used in this method, depending on the required optical density. For highr controls, NANA is added in increasing amounts.

Example II:

Preparation of standard and controls:

NANA 50 mg is dissolved in 10 ml of water (distilled). BSA (bovine

serum albumine) 3-15 g is dissolved in 100 ml of distilled water by stirring.

A proportional amount of NANA is incorporated in the BSA solution to result in a set of concentrations of NANA-BSA reagent which can be used for standard and controls in the test.

The NANA-BSA reagent (standard and controls) is then transferred to vials and lyophilysed. The reconstituted contents of the vials with buffer are used for standard and low and high controls.

The improved reliability, accuracy and reproducibility of the present invention can be seen from the following tables. In table I, showing the results of tests on the blood of normal subjects, the standard deviation of the results using the method of the prior art (US patent 4,748,128 (Katopodis)) is 2.45 while the standard deviation of the results of tests using the method of the present invention is 0.87. In Table II, showing the test results on the blood of patients having cancer, the standard deviation of test results using the method of the prior art is 6.31 while the standard deviation using the method of the present invention is 2.79.

TABLE I

Percent of lipid bound sialic acid in healthy patients (mg per 100 ml)

Present invention		Prior art
21.0		23.4
21.3		23.7
22.0		24.4
19.7		22.1
19.7		22.1
20.8		18.4
22.0		19.6
19.0		16.6
21.0		23.4
21.0		18.6
19.3		18.6
19.9		21.7
21.0		18.6
21.0		23.4
20.0		22.4
19.4		17.0
19.6		22.0
21.3		23.7
21.0		23.4
20.5	mean	21.3
0.870	standard deviation	2.45

TABLE II

Percent of lipid bound sialic acid in cancer patients (mg per 100 ml)

Present invention		Prior art
45.3		41.4
48.4		44.5
52.1		56.0
50.8		54.7
49.8		45.9
44.4		40.5
51.2		55.1
50.6		54.5
50.4		54.3
48.6		44.7
49.9		46.0
52.0		55.9
54.1		58.0
50.5		54.4
56.3		60.2
46.8		42.9
49.4		45.5
53.3		57.2
48.0		44.1
48.0		44.1
49.99	mean	49.99
2.79	standard deviation	6.31

CLAIMS

1. A method of extracting lipid bound sialic acid from human blood plasma or serum and determining the amount of lipid bound sialic acid in a sample of human blood plasma or serum which comprises the following steps:

a) diluting a predetermined volume of a blood plasma or serum sample with an aqueous buffer solution of a volume about 1.5 to 2.5 times that of the predetermined volume of the sample;

b) mixing the diluted sample for a suitable period of time to obtain a substantially homogeneous sample;

c) adding to the sample a mixture of a chlorinated lower alkyl hydrocarbon and a lower alkyl alcohol, the volume of the mixture added being about 20-60 times the predetermined volume of the blood plasma or serum sample, and the volume ratio of chlorinated hydrocarbon to alcohol in the mixture being from about 1:1 to 4:1.

d) mixing the resulting admixture for a suitable period of time to dissolve lipid-bound sialic acid in the sample in the chlorinated hydrocarbon/alcohol mixture;

e) diluting the admixture with a buffer solution at about room temperature, the volume of buffer solution being about 6 to 10 times the predetermined volume of the blood plasma or serum sample;

f) mixing the diluted admixture for a suitable period of time to obtain a substantially homogeneous admixture and centrifuging the mixture to form a substantially clear upper phase;

g) recovering from the clear upper phase so formed a predetermined volume of the upper phase;

h) adding to the predetermined volume of the upper phase a mixture of a protein-precipitating agent and water, the amount of mixture being effective to cause precipitation of the lipid bound sialic acid;

i) mixing the resulting admixture;

j) recovering the resulting precipitate;

k) suspending the precipitate in a hydrolysis agent;

l) determining the amount of lipid bound sialic acid present in the suspended precipitate and thereby the amount present in the blood plasma or serum sample.

2. A method according to claim 1, wherein in step (a) the predetermined volume is about 25 µl and is diluted with about 50 µl of buffer, in step (c) the volume of the added mixture is about 1 ml, and in step (e) the volume of buffer added is about 0.2 ml.

3. A method according to claim 1 or 2, wherein in step (b) the mixing comprises vortexing for at least 2 seconds.

4. A method according to any of claims 1-3, wherein in step (c) the lower alkyl alcohol is methanol or ethanol, and the chlorinated lower alkyl hydrocarbon is chloroform, methylene chloride, ethylene chloride, propylene chloride or tetrachloromethane.

5. A method according to claim 4, wherein in step (c) the lower alkyl alcohol is methanol, the chlorinated lower alkyl hydrocarbon is chloroform, and their volume ratio is about 2:1.

6. A method according to any of claims 1-5, wherein in step (d) the mixing comprises gentle inversion.

7. A method according to claim 1 or 2, wherein in step (f) the mixing comprises gentle inversion for at least 30 seconds followed by centrifuging at above 3500 rpm for at least 5 minutes.

8. A method according to any of claims 1-7, wherein in step (g) the recovering comprises removing the upper phase from the lower phase.

9. A method according to any of claims 1-7, wherein in step (g) the predetermined amount of the upper phase is about 0.2 ml.

10. A method according to any of claims 1-9, wherein in step (h) the protein-precipitating agent is phosphotungstic acid, trichloroacetic acid, ammonium sulfate or a mixture thereof.

11. A method according to claim 10, wherein in step (h) the mixture comprises about 25 ml of phosphotungstic acid and distilled water in a volume ratio of about 1:3.

12. A method according to any of claims 1-11, wherein in step (i) the mixing comprises vortexing for at least 3 seconds.

13. A method according to any of claims 1-12, wherein in step (j) the recovering comprises centrifuging for at least 3 minutes at a speed of about 1300-1600 rpm (250-400 x g).

14. A method according to any of claims 1-13, wherein in step (k) the hydrolysis agent is a resorcinol reagent.

15. A method according to claim 14, wherein in step (l) the amount of lipid bound sialic acid is determined by mixing the precipitate suspended in a volume of the resorcinol reagent, heating at 95-110°C for at least 10 minutes, cooling, adding a volume of a water-immiscible organic solvent selected from alkyl alcohols, alkanoic acid esters and their mixtures, mixing, centrifuging, separating the organic layer, reading at about 580 nm the extracted blue colour present in the organic layer, determining the amount of lipid bound sialic acid by comparing the reading obtained at

580 nm to that obtained for a standard having a known amount of lipid bound sialic acid (LSA) and applying the formula:

$$\text{LSA (mg/100 ml plasma)} = \frac{A \times B}{C}$$

where A = the concentration of the standard; B = the optical density of the sample and C = the optical density of the standard.

16. A method according to claim 15, wherein the volume of resorcinol reagent is about 0.5 ml, the water-immiscible organic solvent is a 85:15 (v/v) mixture of butyl acetate and butanol and its volume is about 0.5-2 times, preferably about equal to, said volume of resorcinol reagent.

17. A method according to claim 15 or 16, wherein the known standard is tested exactly the same as, and simultaneously with, the sample in accord with the method of any of claims 1-16.

18. In a method for determining the amount of lipid bound sialic acid in a sample of human blood plasma or serum involving extraction of lipid bound sialic acid from the sample and precipitation of the lipid bound sialic acid using a protein precipitating agent, the improvement comprising the use of a known standard and the treatment of the standard at the same time and by the same method as the sample with the effect that discrepancies in the test results attributable to variations in the techniques used in the test of the sample and the standard are eliminated.

19. A method according to any of claims 15-18, wherein the standard contains animal blood plasma or serum, human blood plasma or serum, an extract of human or animal tissue, or a proteinous material.

20. A method of diagnosing cancer in a human or animal subject which comprises determining the amount of lipid bound sialic acid in a sample of the subject's blood plasma or serum according to the method of any of claims 1-19 and comparing the amount so determined with values obtained for subjects known to have cancer or with values obtained for normal subjects.

21. A method of diagnosing cancer in a human or animal subject which comprises determining at regular time intervals the amount of lipid bound sialic acid in a sample of the subject's blood plasma or serum according to the method of any of claims 1-19 and comparing the amounts so determined with amounts previously obtained for the same subject.

22. A cancer diagnostic kit comprising a container of a mixture of chlorinated lower alkyl hydrocarbon and lower alkyl alcohol; a container of protein precipitating agent; optionally a container of prediluted resorcinol reagent; optionally a container of a water immiscible organic solvent selected from alkyl alcohols, alkanoic acid esters and their mixtures; a

container of buffer solution; a container of a standard which has a known concentrate of lipid bound sialic acid; and instructions for treating the sample, the standard and the controls.

23. A cancer diagnostic kit according to claim 22, wherein the standard is animal blood plasma or serum or human blood plasma or serum or an extract of human or animal tissue, or a proteinous material.

24. A cancer diagnostic kit according to claim 23, wherein the standard and the controls are in the form of a dry powder.

25. A cancer diagnostic kit according to any of claims 22-24, wherein the lower alkyl alcohol is methanol and the protein precipitating agent is phosphotungstic acid.

26. A method for preparing a standard having a known concentration of lipid bound sialic acid which comprises the following steps:

(a) obtaining a sample of blood plasma or serum from whole blood or a body tissue extract;

(b) diluting a predetermined volume of the sample with distilled water;

(c) mixing the diluted sample to obtain a homogeneous sample;

(d) adding to the sample a lower alkyl alcohol;

(e) mixing the resulting admixture to dissolve matter present in the sample in the alcohol mixture;

(f) treating the admixture to form a supernatant and a precipitate;

(g) diluting the precipitate;

(h) determining the amount of lipid bound sialic acid present in the diluted precipitate or in the supernatant.

27. A method according to claim 26, wherein in step (a) the whole blood is human blood or animal blood and the predetermined volume of plasma or serum is about 100 ml.

28. A method according to claim 26 or 27, wherein in step (b) the sample is diluted by adding to the sample distilled water in a volume which is equal to the predetermined volume of the sample, in step (d) the volume of alcohol added to the sample is about five times the predetermined volume of the sample, and the alcohol is methanol.

29. A method according to any of claims 26-28, wherein in step (f) the admixture is centrifuged at not less than 2500 rpm for about 10 minutes.

30. A method according to any of claims 26-29, wherein in step (g) the precipitate is suspended in a aqueous solution containing up to 150 g serum albumine and optionally up to 3 g N-acetylneuraminic acid (NANA) per liter of distilled water.

31. A method according to claim 30, wherein in step (g) a volume of the aqueous solution which is about equal to the volume of the predetermined volume of the sample is used.

32. A method according to any of claims 26-31, wherein in step (h) the amount of lipid bound sialic acid in the sample is determined by lyophilyzing the precipitate to a dry powder, reconstituting it by adding a buffer solution and comparing the optical density of the solution with the optical density from a standard curve developed from a standard sample of N-acetylneuraminic acid (NANA).

33. A method according to any of claims 26-29, wherein in step (h) the amount of lipid bound sialic acid in the sample is determined by condensing the supernatant to about 1/10 of its volume, purifying the supernatant by extraction with a chloroform-methanol mixture, partitioning the mixture with water, treating the resultant solution so as to obtain its optical density, and comparing said optical density with that of a standard sample of N-acetylneuraminic acid (NANA).

34. A method for preparing a standard having a known concentration of lipid bound sialic acid which comprises the following steps:

(a) dissolving a proteinous material in distilled water in an amount of 3-15 g per 100 ml;

(b) optionally adding up to 0,5 g of N-acetylneuraminic acid to the solution of the proteinous material in water;

(c) determining the amount of lipid bound sialic acid present in the resulting mixture.

35. A method according to claim 35, wherein in step (c) the resulting mixture is lyophilysed, reconstituted by adding a buffer solution and comparing the optical density of the reconstituted solution with the optical density from a standard curve developed from a standard sample of N-acetylneuraminic acid (NANA).

36. A method according to claim 35 or 36, wherein the proteinous material is a protein having a molecular weight of between 10,000 and 100,000, and is preferably a serum albumine.

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 91/00233

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 G01N33/50; G01N33/96; G01N33/50; G01N33/92 //G01N33/574

II. FIELDS SEARCHED	
Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	G01N
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]	

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	US,A,4 342 567 (N. KATOPODIS) 3 August 1982 cited in the application see the whole document	1,2
A	US,A,4 748 128 (N. KATOPODIS) 31 May 1988 cited in the application see the whole document	1,2
A	PROCEEDINGS OF AACR AND ASCO vol. 21, March 1980, SAN DIEGO, CALIFORNIA, US page 182; N. KATOPODIS ET AL: 'Spectrophotometric assay of total lipid bound sialic acid in blood plasma of cancer petients and asymptomatic healthy individuals.' cited in the application see abstract	1,2

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 FEBRUARY 1992	18. 03. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	HITCHEN C.E.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	US,A,4 146 603 (E.A.DAVIDSON ET AL) 27 March 1979 cited in the application	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9100233 SA 53743

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/02/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4342567	03-08-82	None		
US-A-4748128	31-05-88	AU-B-	587777	31-08-89
		AU-A-	4041485	03-10-85
		CA-A-	1253778	09-05-89
		EP-A,B	0159564	30-10-85
		JP-A-	60259961	23-12-85
US-A-4146603	27-03-79	CA-A-	1087979	21-10-80
		CH-A-	648330	15-03-85
		CH-A-	658317	31-10-86
		DE-A,C	2806146	24-08-78
		FR-A,B	2381058	15-09-78
		GB-A-	1591561	24-06-81
		JP-C-	1368233	11-03-87
		JP-A-	53127822	08-11-78
		JP-B-	61029464	07-07-86
		JP-C-	1411736	27-11-87
		JP-A-	60155975	16-08-85
		JP-B-	62018869	24-04-87